

The determination of Q192R polymorphism of paraoxonase 1 by using non-toxic substrate p-nitrophenylacetate

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CONTEXT: The human serum paraoxonase 1 (PON1) is calcium-dependent esterase and associates with the high density serum lipoproteins. PON1 plays a major role in oxidation of high density lipoprotein and low density lipoprotein and prevention of atherogenesis in coronary heart disease. PON1Q and R allele hydrolyses number of substrates like paraoxon (PO) (diethyl p-nitrophenyl phosphate) and phenylacetate.

AIMS: The aim of the study is to the determination of Q192R polymorphism of PON1 by using non-toxic substrate p-nitrophenylacetate and compares it with the phenotype determined by using PO as substrate.

MATERIALS AND METHODS: The study group consists of 60 healthy normal patients. Paraoxonase activity was measured using the procedure described by Eckerson (Reference method) and for phenotyping; the ratio of hydrolysis of PO in the presence of 1 M NaCl (salt-stimulated PON1, SALT) to the hydrolysis of phenylacetate (PA) is calculated. In new method (Haagen *et al.*) arylesterase activity measured using p-nitrophenylacetate and for phenotyping arylesterase, the ratio of inhibition of enzymatic hydrolysis of p-nitrophenylacetate (substrate) by phenyl acetate to non-inhibited hydrolysis of p-nitrophenylacetate (inhibited arylesterase activity (IA-IA₀)/non-inhibited arylesterase activity (NIA).

RESULTS: It was found that paraoxonase activity is trimodally distributed in both the methods. There is no significant difference in the distribution of PON1 phenotypes of both reference method and new method being frequencies 0.946 and 0.376 respectively and there was no significant difference for phenotypic polymorphism for an individual by both methods ($\chi^2 = 0.15$ and $P = 0.9262$).

CONCLUSION: The Q192R polymorphism of PON1 by using non-toxic substrate p-nitrophenylacetate showed trimodal distribution of QQ (homozygous), QR (heterozygous), and RR (homozygous) phenotype and it is comparable with reference method. This method can be used for PON1 phenotype in different pathological and complex disease conditions.

Key words: Paraoxonase 1 polymorphism, arylesterase, p-nitrophenylacetate, paraoxon

Introduction

Serum paraoxonase 1 (PON1) is calcium-dependent esterase belongs to the paraoxonase family, which is widely distributed among tissues such as liver, kidney, and intestine and is located in the chromosomal region 7q21.3-22.1.^[1,2] PON1 is synthesized in the liver and that hydrolyses organophosphate insecticides and nerve gases.^[3] PON1 is a protein of 354 amino acids with a molecular mass of 43 kDa.^[4,5] In serum, it is almost exclusively located on high density lipoprotein (HDL).^[6] Paraoxonase has shown to prevent low density lipoprotein (LDL) and HDL oxidation both *in vivo* and *in vitro* through hydrolysis of lipid peroxides. The oxidative change of LDL is one of the major events of early atherogenesis that contributes to cholesterol accumulation in the arterial wall and lesion development.^[7,8] It has been shown that paraoxonase has protective effect on coronary heart disease and related conditions such as myocardial infarction,^[9] Stroke,^[10] insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM),^[11,12]

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hypercholesterolemia,^[12] chronic renal failure,^[13] some neurological conditions like Alzheimer's disease^[14] and parkinsonism,^[15] some of the tumors^[16,17] and organophosphorus poisoning.^[18]

Human PON1 has various polymorphic sites among which one of them site giving rise to amino acid substitution at position 192, glutamine (Q) to arginine (R), which is significantly affecting the catalytic efficiency of PON1, in a substrate-dependent manner and most exclusively studied.^[19] The PON1 Arg (R) allele is much more efficiently hydrolyze PO than PON1Glu (Q), determining a triphasic distribution of serum paraoxonase activity in Caucasian population.^[20] This allelic polymorphism has been studied in number of diseases. In recent years, research has been directed toward evaluating the roles of the different polymorphic forms in atherosclerosis and vascular disease, due to differential abilities of the 192R and Q isoform in preventing oxidation of LDL. The HDL from QQ homozygote was found to be more protective of LDL than RR homozygotes which are least effective, because the Q allozyme provides greater protection against the accumulation of lipid peroxides on LDL than the R allozyme.^[21,22]

PON1 possess both arylesterase and paraoxonase activity. PON1 hydrolyses number of substrates like PO (diethyl p-nitrophenyl phosphate), phenylacetate, 2-naphthyl acetate, p-nitrophenylacetate, chlorpyrifos oxon, diisopropyl fluorophosphate and 4-chloromethyl phenylacetate.^[23-27] In 1983, Eckerson studied PON1 Q192R phenotypic polymorphism by using PO as substrate (reference method) in which basal and salt-stimulated (NaCl) arylesterase and phenylacetate activities determined^[20] whereas Haagen *et al.* used p-nitrophenylacetate and phenylacetate as substrate (new method) in which non-inhibited arylesterase activity (NIA) and inhibited arylesterase activity (IA) determined.^[28]

For determination of phenotypic studies of PON1, PO is used however, PO exposure can cause rapid, severe organophosphate poisoning with headache, dizziness, sweating, nausea and diarrhea, loss of coordination, muscle twitching, convulsions, coma and death. Breathing PO can irritate the nose, throat, and lungs. It can also affect concentration, memory, vision,

and muscle coordination.^[29] Furthermore, during assay safety measures has to be taken like use of face mask and double gloves, preparation of required quantity of working reagent in one setting of analysis and treatment of waste water receptacle and pipettes with 2 N NaOH. PO is not compatible with strong bases such as NaOH and hence it has to be dispose in NaOH. It has been shown that instead of PO, p- nitrophenylacetate can be used for PON1 phenotyping. The aim of our study to determine Q192R phenotypic polymorphism by using p-nitrophenylacetate as substrate and compare or correlate with the phenotype defined by the Eckerson's method.

Materials and Methods

The study group consisted of 60 healthy normal patients came to outpatient department of our hospital with the age ranging from 30 years to 60 years. Written valid informed consent was obtained from all subjects. Individuals with the history of ischemic heart disease, diabetes mellitus, hypertension, hyperlipidaemia and chronic renal failure were excluded from the study. The study was approved by the institutional ethical committee.

Paraoxonase activity

Paraoxonase activity was measured by adapting the procedure described by Eckerson *et al.* The reaction mixture contained 1.0 mmol/L (milimoles per litre) diethyl p-nitrophenyl phosphate (PO), 1.0 mmol/L CaCl_2 and 100 mmol/L tris-HCl buffer, pH 8.0, with or without 1 mol/L NaCl. One unit of paraoxonase activity produces 1 nmol of p-nitrophenol and the activity is expressed as U/L based on the molar absorption coefficient (18050 M/cm) at 405 nm at pH 8.0.^[20]

Arylesterase activity with phenyl acetate as substrate

Serum arylesterase catalyzes hydrolysis of phenylacetate to form phenol. The rate of hydrolysis of phenylacetate is assessed by measuring the liberation of phenol at 270 nm. The reaction mixture contains 4.0 mmol/L phenylacetate, 1 mmol/L CaCl_2 dissolved in 20 mmol/L tris-HCl buffer, pH 8.0 at 25°C. The activity is expressed as kU/L, based on the extinction coefficient

of phenol of (1310 M/cm) at 270 nm, pH 8.0, and 25°C after correction for non-enzymatic hydrolysis.^[20]

Arylesterase activity with p-nitrophenyl acetate as substrate and inhibition of p-nitrophenylacetate hydrolysis by phenylacetate

The reaction mixture or working reagent consisted of 25 mmol/L triethanolamine-hydrochlorine buffer, pH 7.4, with 1mmol/L CaCl_2 , 2.5 mmol/L p-nitrophenyl acetate in water with (IA) or without 1 mmol/L phenyl acetate (NIA). The activity expressed in kU/L, based on the molar absorptivity (14,000) of p-nitrophenol at 405 nm, at pH 7.4.^[28]

Ratios of enzyme activity

After estimating paraoxonase and arylesterase activity, we calculated enzymatic ratios: (i) The salt-stimulation ratio (SALT/PA) was defined as the salt-stimulated paraoxonase activity (SALT) over arylesterase activity, with PA as substrate, (ii) The inhibition ratio $(\text{IA}-\text{IA}_0)/\text{NIA}$ was defined as the PA-IA with p-nitrophenyl acetate as substrate minus the estimate of influence of nonspecific arylesterase activity of other carboxylic ester hydrolases (IA_0) divided by the NIA with p-nitrophenylacetate as substrate.^[20,28]

Statistical analysis

Statistical data were analyzed with MYSTAT student version. The observed allele frequencies were determined by Hardy-Weinberg equilibrium and are evaluated by the χ^2 test. Phenotype comparison was assessed by

Mecnemar (χ^2) statistic test. Values of $P < 0.005$ were considered to be significant.

Results

In the Figure 1a and 2a, showing graph of the ratio of inhibited to non-inhibited arylesterase versus frequency of individuals, it shows the trimodal distribution of the study population with the two antimodes. According to these antimodes the study group is distributed into 21 individuals with QQ phenotype, 27 individuals with QR phenotype and 12 individuals with RR phenotype with allele frequency of allele-Q and allele-R being 0.575 and 0.425 respectively. In Figure 1b and 2b, showing graph of the ratio of salt stimulated paraoxonase activity to arylesterase activity versus frequency of individuals; it shows the trimodal division of the study population by the two antimodes. According to these antimodes the study group is distributed into 23 individuals with QQ phenotype, 26 individuals with QR phenotype and 11 individuals with RR phenotype with allele frequency of allele-Q and allele-R being 0.625 and 0.375 respectively. In both the methods phenotype distributions were comparable and also consistent with published reports of frequencies in Caucasians using a method based on PCR. In the Figure 3, shows graph of ratio of reference vs new method which separates 3 different phenotypes.

There is no significant difference in the distribution of PON1 phenotypes of both reference method and new method being frequencies 0.946 and 0.376 respectively. Table 1 shows the distribution of PON1 phenotype and allele frequency determined by using p-nitrophenyl

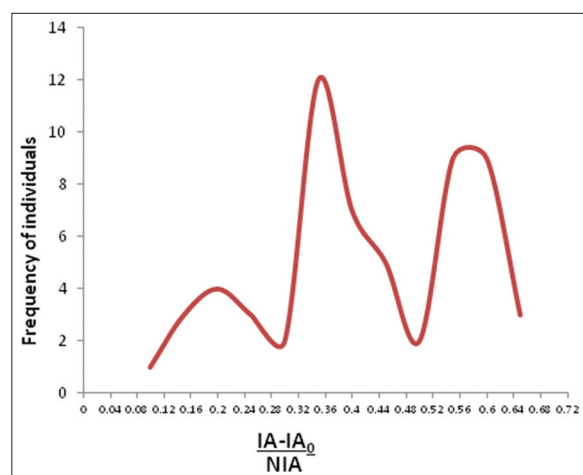


Figure 1a: Trimodal population distribution of PON1 phenotype by using p-nitrophenylacetate

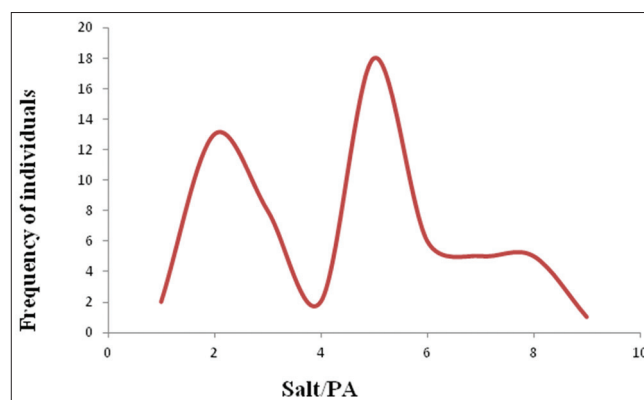


Figure 1b: Trimodal population distribution of PON1 phenotype by using paraoxon (PO)

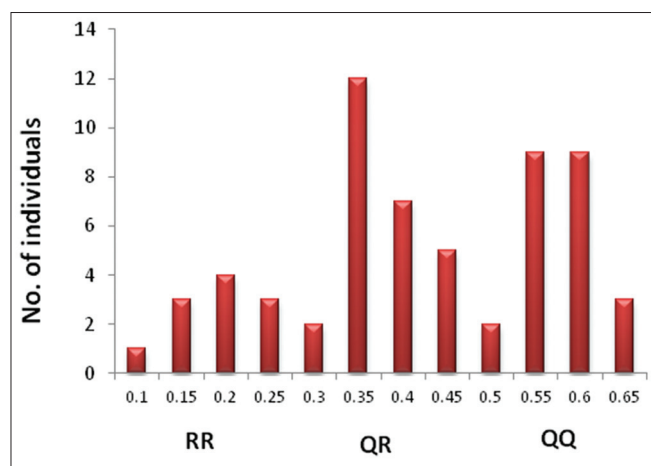


Figure 2a: Population distribution of PON1 phenotype by using p-nitrophenylacetate

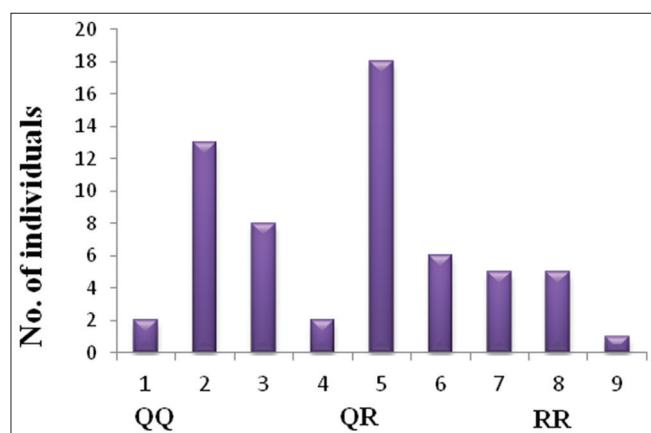


Figure 2b: Population distribution of PON1 phenotype by using paraoxon (PO)

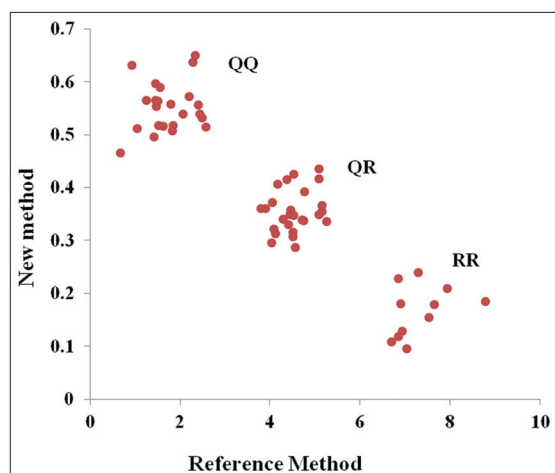


Figure 3: Ratio of reference vs new method

acetate as substrate while Table 2 shows the distribution of PON1 phenotype and allele frequency determined by using paraoxon as substrate. By Mecnemar test (Chi-square), there was no significant difference

Table 1: Distribution of PON1 phenotype and allele frequencies determined by using p-nitrophenylacetate as substrate

Observed no. of individuals with each phenotype	Observed allele frequencies
QQ=21 (35%)	Allele Q=0.575
QR=27 (45%)	Allele R=0.425
RR=12 (20%)	
Total=60	

Table 2: Distribution of PON1 phenotype and allele frequencies determined by using paraoxon as substrate

Observed no. of individuals with each phenotype	Observed allele frequencies
QQ=23 (38.33%)	Allele Q=0.625
QR=26 (43.33%)	Allele R=0.375
RR=11 (18.33%)	
Total=60	

for phenotypic polymorphism for an individual by both methods ($\chi^2 = 0.15$ and $P = 0.9262$).

Discussion

In both the new and reference method it was clearly described that the study population divides into 3 different (trimodal distribution) groups. In our study, it was shown that distribution of PON1 phenotype by new method (Haagen *et al.*) is nearly same as that of distribution among method defined by Eckerson *et al.* It was not possible to confirm correct classification or observed distribution of PON1 phenotype with that of PCR genotyping however; we have compared phenotype frequency with that of standard reference method. Phenotype determined by the $(IA-IA_0)/NIA$ method misclassified two of the QQ as QR and one QR as RR. The area of overlap between QR and RR distribution appeared to be slightly more in the SALT/PA than in the $(IA-IA_0)/NIA$. According to Eckerson *et al.*^[20] the frequency of Q (previously A) allele is 0.685 and that of the R (previously B) allele is 0.315 in a population of Caucasians. The allele frequency in the present study are quite near to these figures in both $(IA-IA_0)/NIA$ and SALT/PA which is 0.425 for R allele and 0.575 for Q allele and 0.375 and 0.625 respectively.

PON1 is an extensively studied enzyme, particularly for its role in hydrolyzing a huge number of OPs. There is wide variation in serum PON1 levels and activity among

different ethnic populations. This is believed to be so because PON1 exists in different polymorphic forms. The Q192R polymorphism has been found to affect the rates of hydrolysis toward various substrates including PO, diazoxon, soman, and sarin. The allele with Arg at position 192 (PON1R192) efficiently hydrolyses PO at a higher rate than the allele with Glu at this position (PON1Q192). However, the R allele is 8 times less efficient in hydrolyzing 3 types of OPs, namely diazoxon, sarin and soman while Q allele hydrolyses these substrates more quickly.^[30-32] Most studies investigating the association of PON1 polymorphisms with diseases have examined only the nucleotide polymorphisms (Q192R, L55M, C-108T) with PCR-based assays and ignored the plasma PON1 levels, the most important determinant of the rates of metabolism of endogenous or exogenous toxins. However, even if an individual were genotyped for all known PON1 polymorphisms, this analysis would not provide the level of plasma PON1 activity nor the phase of polymorphisms (i.e., which polymorphisms are on each of an individual's two chromosomes).^[33] Evaluation of only genetic polymorphisms as risk factors in complex diseases is inadequate. Many authors have emphasized the importance of measurement of PON1 activity and/or concentration in addition to PON1 genotypes, when exploring the role of PON1 in vascular disease.^[34-36] The term PON1 status, which includes both PON1 192 genotype and PON1 activities, supplies much broader information than the genotype alone, for correlation with disease susceptibility, or responsiveness to environmental agents. It stands to reason that higher levels of a protein involved in metabolizing oxidized lipids or toxic OP compounds would be more protective and the level of protection should be related to the activity level of the protein.^[37] Studies that examined either PON1 levels or PON1 status (plasma PON1 activity levels and Q192R genotype) have shown that low PON1 levels are a risk factor for vascular disease,^[38] whereas some studies shown that there was no association observed PON1 genotype.^[34,36] In addition there are number of studies on genotype and phenotype of PON1 polymorphism. As the PON Q192R genetic polymorphism has been suggested to be an independent risk factor for coronary artery disease, PON1Q plays an important role in the protection of LDL and HDL oxidation than PON1R.^[22]

For the 1st time Ruiz *et al.*^[39] have shown that the B (R) and AB+B (QR+RR) genotype were associated with an increased risk of CHD in NIDDM patients and hypothesized that the activity of the B (R) phenotype does not protect against lipid peroxidation. Furthermore, the similar results have been shown in Japanese diabetic patients, US and Indian CHD patients. In contrast recent study by Flekac *et al.* demonstrated that the presence of PON1 QQ genotype was associated with poorer diabetes control than RR genotype.^[40] A large number of studies have shown similar PON1 polymorphism findings. Lee *et al.* studied the risk of developing lung cancer was significantly increased in individuals carrying the PON1 QQ genotype.^[41] Dantoine *et al.* concluded from their study that PON1 polymorphism 192 may be considered to be a reliable marker to distinguish patients with Alzheimer's disease from patients with vascular dementia and from healthy subjects.^[14] Kucukali *et al.* conducted a study to evaluate the association between PON1 polymorphism and Schizophrenia suggesting that the subjects carrying R allele or RR genotype might be susceptible to Schizophrenia and subjects with QQ might be protected against Schizophrenia.^[42]

The phenyl acetate and PO are the two substrates used for the arylesterase (paraoxonase) phenotyping in number of studies published. As previously described, the pure form of PO is the neurotoxic substance. Thus it may be concluded that, the p-nitrophenyl acetate can be used instead of PO as a substrate for differentiating the isoform. The new method is an easy and non-toxic method for determination of Q192R polymorphism in various pathological conditions such as CHD, NIDDM and chronic renal failure etc. Furthermore, both genotyping and purification of Q and R types of PON1 require complex, expensive procedures. Therefore in our study, it is clear that PON1 phenotype in both the new and reference method are comparable and reference (Eckerson's) method can be replaced by the new method.

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